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An Acidic Fibroblast Growth Factor Protein Generated by Alternate Splicing Acts Like an Antagonist

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Summary

Polymerase chain reaction amplification of cDNA for acidic fibroblast growth factor in several lines of cultured human cells revealed two forms of mRNA. The novel smaller mRNA lacks the entire second coding exon of the acidic fibroblast growth factor gene, whereas the previously identified mRNA consists of three coding exons. The truncated variant of acidic fibroblast growth factor (aFGF') is only 60 amino acids long with an apparent molecular mass of 6.7 kD on sodium dodecyl sulfate gels in contrast to 18 kD for the full-length acidic fibroblast growth factor. aFGF' elicits only minimal fibroblast proliferation and antagonizes the effects of acidic fibroblast growth factor when added exogenously to or when coexpressed with aFGF in BALB/c/3T3 fibroblasts. Thus, the truncated variant of acidic fibroblast growth factor may provide fibroblasts with a unique mechanism for endogenous regulation of their responses to acidic fibroblast growth factor.

The fibroblast growth factor (FGF)¹ family presently consists of seven different proteins that have mitogenic and chemotactic activities for a wide variety of mesoderm- and neuroectoderm-derived cells (1). Acidic FGF (aFGF) and basic FGF (bFGF) are expressed in normal tissues as well as tumors, where they contribute to wound healing and angiogenesis, and may have important roles in cellular development and physiology (1–5). Although the mechanisms of release of aFGF and bFGF from cells have not been defined, they act specifically by binding to high-affinity cellular receptors. FGF receptors belong to the tyrosine kinase family of receptors (6), which are activated by FGF binding and result in the phosphorylation of tyrosine residues in the receptor cytoplasmic domain and other cellular substrates (7). Another consequence of the activation of FGF receptors is a dramatic increase of expression of the protooncogene *c-fos* (8). Two members of the FGF family, *int-2* (9) and *hst/Ks3* (10), are products of putative oncogenes, and aFGF and bFGF also have transforming potential as demonstrated by the effects of transfection of the respective genes into fibroblasts (11). The genes for FGFs are highly conserved among species and are similar in their overall organization of three coding exons separated by two large introns. In the case of aFGF, two additional upstream non-

coding exons have been identified recently that are alternatively spliced to the first coding exon (12). In addition, multiple polyadenylation sites lead to a highly heterogeneous aFGF mRNA pattern (13).

In the course of our studies, which aimed at the analysis of the expression of aFGF in several human cell lines with the help of the reverse transcription polymerase chain reaction (RT-PCR) methodology, we discovered that next to the expected cDNA made up of all three coding exons, an additional smaller cDNA is amplified that is missing the entire second coding exon of the aFGF gene. The alternate aFGF mRNA encodes a 60-amino acid protein that we termed aFGF'. We describe here our studies with synthetic aFGF', which indicate that the newly found alternate form acts like an antagonist for aFGF.

Materials and Methods

Cell Culture. Human lung fibroblasts (obtained from a lung biopsy), human dermal fibroblasts CRL 1564, human medulloblastoma TE671 cells, and murine BALB/c/3T3 fibroblasts (UCSF Tissue Culture Facility) were cultured as monolayers in DME H21 supplemented with 10% heat-inactivated FCS, 4.5 g/liter glucose, 25 mM Hepes, 10⁵ U/liter penicillin-G, and 10⁵ U/liter streptomycin sulfate. Human umbilical vein endothelial cells (Clonetics, San Diego, CA) were cultured in growth medium EGM-UV (Clonetics). Cells were grown at 37°C in 5% CO₂ in air and split at 1:4 after they had reached confluency.

¹ Abbreviations used in this paper: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; RP, reversed-phase; RT, reverse transcription.

Isolation of RNA, RT, and PCR. Total cytoplasmic RNA was isolated from 10^5 human lung fibroblasts and reverse transcribed with an oligo(dT) primer (Pharmacia LKB, Piscataway, NJ). PCR amplification of the resultant single-stranded cDNA mixture was carried out with primers 5'CGGGAATCTCTTGAAAGCGCCA-CAAGCAGCAG 3' and 5'ACGAGAATCTCTGGAGTGGTCA-ACACCCA 3', which include EcoRI adaptor sites at their ends and recognize the flanking sequence ends of a 531-bp cDNA encoding the entire human aFGF. The PCR reaction mix consisted of 58.5 μ l of sterile double-distilled water, 10 μ l of $10\times$ reaction buffer, 16 μ l of 1.25 mM dNTP mix, 0.5 μ l of Taq polymerase (Promega Corp., Madison, WI), 5 μ l of each of the primers at 20 μ M, and 5 μ l of single-stranded cDNAs. Each of the 35 cycles performed in a PCR thermocycler (Perkin Elmer Cetus, Norwalk, CT) included denaturation at 94°C for 30 s, reannealing of primers and fragments at 55°C for 1 min, and primer extension at 72°C for 2 min. The resultant cDNA products were analyzed on a 3% agarose gel.

Cloning and Sequencing. DNA fragments amplified by PCR were purified on agarose gel and electroeluted, repaired with Klenow, kinased, and cloned into a SmaI- (or EcoRI)-digested and phosphatase-treated Mp13M18 vector. DNA sequencing was performed on single-stranded M13 DNA by the dideoxy chain termination method.

Bacterial Expression of aFGF'. aFGF' was expressed as a protein A fusion protein using the pRIT5 vector (Pharmacia LKB) in *Staphylococcus aureus* according to the manufacturer's instructions. After isolation of the fusion protein with the help of an IgG-Sepharose column (Pharmacia LKB), the purified protein was subjected to cyanogen bromide cleavage (14), which results in an aFGF' form that is lacking the first methionine residue. Final purification of aFGF' (amino acids 2–60) was carried out by reversed-phase high performance liquid chromatography (RP-HPLC) and peptide identity confirmed by gas-phase protein sequence analysis.

Peptide Synthesis. The aFGF' protein was chemically synthesized with a peptide synthesizer (431; Applied Biosystems, Inc., Foster City, CA). Amino acids were coupled as their FMOC derivatives (Bachem, Torrance, CA). Peptides were deprotected and cleaved off the resin with TFA/anisole/dimethylsulfide/ethanedithiol (93:3:3:1) and purified by RP-HPLC. Peptide identity was confirmed by gas-phase sequence and mass spectrometric analyses.

Western Blot. The polyclonal antiserum for the immunoblot was prepared by immunizing rabbits with synthetic aFGF', or was obtained from a commercial source (directed against epitopes derived from exons 2 and 3 of the aFGF gene; Promega Corp.). Proteins extracted from the nuclear fraction of 10^8 confluent human lung fibroblasts with SDS-PAGE sample buffer were resolved on 14% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes bearing blot-transferred proteins were soaked in a blocking buffer of 5% nonfat dry milk in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, for 2 h at room temperature and then incubated with antiserum or preimmune serum at a 1:500 dilution in blocking buffer. IgG bound to transferred proteins was detected with the alkaline phosphatase system, as described by the manufacturer (Promega Corp.).

Mitogenesis Assays. The mitogenic effects of aFGF and aFGF' (recombinant as well as chemically synthesized forms of the protein were used and had identical activities) alone and in combination were determined by measuring their ability to stimulate uptake of [3 H]thymidine by BALB/c/3T3 cells. 5×10^3 BALB/c/3T3 cells were seeded into each well of 96-well plates, grown to confluence in culture medium, starved for 7 d, and then stimulated with either human recombinant aFGF (Promega Corp.) or recom-

binant aFGF' for 24 h, followed by addition of 1 μ Ci of [3 H]thymidine (Amersham Corp., Arlington Heights, IL) to each well. After 10 h of further incubation, the cells were detached and dispersed with trypsin, deposited onto individual glass fiber filters for determination with a cell harvester of incorporated [3 H]thymidine in a scintillation counter.

Affinity Crosslinking of 125 I-aFGF' to Fibroblast Receptors. BALB/c/3T3 cells were grown to confluence in 60-mm wells, and washed twice with 10 ml of cold in PBS before addition of 1 ml of binding buffer (DME H21 with 25 mM Hepes, pH 7.5, 0.2% gelatin) and 125 I-aFGF' (2×10^6 cpm). Control wells designed to assess non-specific labeling also received a 100-fold excess of unlabeled aFGF'. 2 ml of disuccinimidyl suberate, which had been diluted from a concentration of 20 mM in DMSO to 0.15 mM in PBS at room temperature, was added to each well. After crosslinking at room temperature for 15 min, the incubation medium was aspirated, each well was washed twice with 10 ml of cold PBS, and the cells were removed with a pipette tip in 200 μ l of SDS-PAGE sample buffer. The cellular lysates were incubated at 4°C for 10 min, boiled for 5 min, and a 50- μ l portion was loaded onto a 7.5% SDS-polyacrylamide gel and subjected to electrophoresis. The gels then were soaked in EN 3 HANCE (Amersham Corp.), dried, and autoradiographed.

Binding Studies. 4×10^4 BALB/c/3T3 cells were seeded into 16-mm-diameter wells and grown to confluency. The adherent cells were washed once with 0.25 ml of cold PBS and incubated in 0.5 ml of the binding buffer (see above) for 60 min at 4°C with various amounts of 125 I-aFGF, radiolabeled with iodobeads (Pierce Chemical Co., Rockford, IL) and Na 125 I (Amersham Corp.), with or without 10 μ g of unlabeled aFGF'.

Analysis of c-fos Expression. BALB/c/3T3 cells were grown to confluency and starved in serum-free medium for 48 h. The cells were then incubated with either aFGF (50 ng/ml), aFGF' (18.6 ng/ml), or medium alone for 10, 20, and 30 min. RNA was extracted from each suspension of cells by the guanidinium isothiocyanate (GIT)-CsCl ultracentrifugation method, and 10 μ g of RNA was each fractionated on a 1% agarose gel containing 2.2 M formaldehyde in $1\times$ 3-(N-morpholino) propanesulfonic acid (MOPS) buffer, and transferred to a nitrocellulose filter in $10\times$ SSC. The filter was probed with 100 ng of a randomly primed 32 P-labeled c-fos cDNA fragment (generated by PCR with c-fos-specific primers) and washed once for 20 min at room temperature in $1\times$ SSC, 0.1% SDS, and three times for 20 min each at 68°C in $0.2\times$ SSC, 0.1% SDS, dried, and exposed to x-ray film at -70°C .

Generation of Stable Transfectants. The coding sequences for aFGF and aFGF' were cloned into the mammalian transfection vector pRC/CMV (Pharmacia LKB) and the recombinant plasmids transfected into BALB/c/3T3 cells by the lipofection (Bethesda Research Laboratories, Gaithersburg, MD) procedure. Briefly, 10 μ g of the plasmid was mixed with the lipofectin reagent, and after 15 min incubation time at room temperature, the DNA lipofectin mixture was mixed with 3T3 cells in a FCS-free optimen medium (Bethesda Research Laboratories). After 24 h, the optimen media was removed and replaced with DME and 10% FCS. For selection, 500 μ g/ml geneticin antibiotic (Gibco Laboratories, Grand Island, NY) was added to the culture, and incubation continued for 2 wk, at which time geneticin-resistant colonies were visible. Individual colonies were then transferred to 17-mm wells and grown up. All transfectants were assessed for their mRNA levels using a ribonuclease protection assay (see below).

Ribonuclease Protection Assay. An RNA probe complementary to the entire coding region of the aFGF gene was used and was generated by transcription from the pbCIKS $^+$ plasmid (Strata-

gene, La Jolla, CA) in the presence of [32 P]dUTP. RNA synthesis was carried out with T3 polymerase and the RNA purified on a 5% preparative polyacrylamide gel. 10^5 cpm of the labeled RNA probe was then coprecipitated with 50 μ g total RNA, the RNA pellet dissolved in 30 μ l of 80% formamide, 400 mM NaCl, 40 mM Pipes (pH 6.4), and 1 mM EDTA (pH 8). After heating at 85°C for 10 min, hybridization was performed at 42°C for 12 h. Subsequently, 297 μ l of 1 \times RNase buffer (5 ml 300 mM sodium acetate, pH 7, 0.5 ml 10 mM Tris-HCl, pH 7.5, 0.5 ml 5 mM EDTA, pH 8) was added along with 1 μ l of RNase A (10 mg/ml) and 2 μ l of RNase T₁ (10 mg/ml). After a 30-min incubation at 30°C, 6.6 μ l of 10% SDS and 4.4 μ l of proteinase K (10 mg/ml) were added and incubated for 15 min at 42°C. The mixture was then extracted with phenol/chloroform/isoamylalcohol, and the protected RNA was precipitated by adding 1.5 μ l tRNA (10 mg/ml) and 800 μ l ethanol. The pellet was dissolved in 30 μ l of 80% formamide, 1 \times TBE, 1 mM EDTA, 0.5% bromophenol blue, and 0.05% xylene cyanol, denatured at 95°C for 5 min and resolved on a 5% polyacrylamide gel. Dried gels were exposed to x-ray film at -70°C for 12 h. To assess the ratio of aFGF' and aFGF mRNA in the cotransfectant cell lines, gel pieces corresponding to the three protected RNA fragments were cut out, and radioactivity was measured with a scintillation counter.

Proliferation Assay. On day 0, 30,000 cells were seeded into 60-mm dishes in DME supplemented with 5% FCS. After 5 d, triplicate dishes were trypsinized, and the cell number was assessed with a cell counter (Coulter Electronics, Inc., Hialeah, FL).

Results

Analysis of aFGF Expression. PCR amplification of cDNA templates derived from mRNA of different human cultured cells (lung fibroblasts, dermal fibroblasts, and medulloblastoma cells), with oligonucleotide primers specific for the aFGF gene, yielded two cDNAs of distinct sizes (Fig. 1). One cDNA was ~500 bp, expected for aFGF, and the other cDNA was ~100 bp smaller. Only the previously identified 531-bp cDNA was cleaved by PstI into the predicted fragments of 212 and 319 bp, whereas the smaller cDNA species appeared to lack the unique PstI cleavage site located within exon 2 of the aFGF gene (data not shown). Complete nucleotide sequence analysis confirmed the absence of the entire second

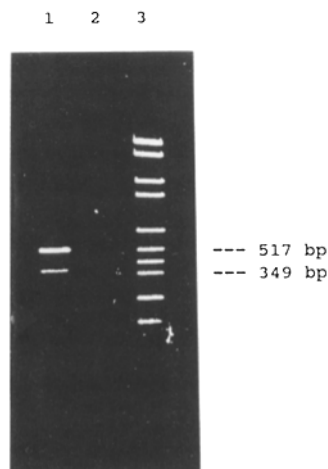


Figure 1. Expression of aFGF in human lung fibroblasts. Agarose gel electrophoresis of RT-PCR amplification products (see Materials and Methods). Two cDNA species were amplified from human lung fibroblasts with specific oligonucleotide primers flanking the coding region of the aFGF gene. Lane 1, lung fibroblasts cDNA template; lane 2, negative control (no template); lane 3, DNA molecular mass markers.

exon of the aFGF gene from the smaller aFGF cDNA, which was designated aFGF' (Fig. 2). Further evidence that the above cells expressed an mRNA species lacking the second coding exon was obtained by a ribonuclease protection assay with an antisense aFGF RNA probe that encompassed the entire coding sequence of the aFGF gene. Next to the expected full-length aFGF mRNA (536 bp), the probe also protected two RNA species that represented fragments corresponding to the first (230 bp) and third (203 bp) coding exons of the aFGF gene and were derived from the aFGF' mRNA (Fig. 3).

The lack of the second exon in the smaller aFGF' mRNA, which is presumably the consequence of alternative splicing, shifts the reading frame to create a premature termination of translation 12 nucleotides downstream from the beginning of the third exon. The loss of 104 coding base pairs of exon 2 combined with an early termination of translation yields an alternate aFGF' protein, which is only 60 amino acids long, of which, 56 are identical to the NH₂-terminal end of aFGF (Fig. 2).

Analysis of aFGF' Activities. For functional and biochemical analyses, both recombinant and chemically synthesized aFGF' were prepared (see Materials and Methods). Synthetic and recombinant aFGF' had identical activities in all assays (see below) and also served as the immunogen to generate polyclonal antibodies in rabbits. Western blots of nuclear extracts from quiescent human lung fibroblasts were probed with the polyclonal antiserum raised against aFGF' (Fig. 4). Besides several nonspecific high molecular mass proteins in both lanes, two proteins were detected by the immunoserum (Fig. 4, lane 2), but not preimmune serum (Fig. 4, lane 3), which were competed by excess aFGF' peptide (data not shown) and have sizes of 18 and 7 kD. These are presumed to be aFGF and aFGF', respectively, since they are comigrating with human recombinant aFGF and aFGF' on SDS gels (Fig. 4, lane 1). Western blots carried out with commercial antisera (see Materials and Methods) directed against aFGF epitopes encoded by exons 2 and 3 showed only the 18-kD protein signal, which argues against the possibility that the 7-kD protein is a degradation product of aFGF (data not shown). The newly discovered variant of aFGF mRNA hence codes for a protein of the size expected for a deletion of the second exon and a premature stop codon. Although it appears that the signal for aFGF is more intense than that for aFGF', it is difficult to assess the exact ratio of the two proteins using this type of analysis. Attempts to detect immunoreactive proteins in total cellular extracts failed, presumably due to the small amounts of material. Only enrichment of the nuclear fraction yielded sufficient aFGF and aFGF' that could be detected with the above method.

Since aFGF is a very potent mitogen for a wide range of mesodermal and neuroectodermal cells (1), mitogenesis assays were performed to examine the biological activity of the alternate aFGF'. aFGF' has only a minimal mitogenic potential for fibroblasts as compared with the commercial full-length aFGF (half-maximal activity in the absence of heparin is 20 ng/ml), when assessed by increases in the uptake of [3 H]thymidine into murine BALB/c/3T3 (Fig. 5 A) and human umbilical vein endothelial cells (data not shown).

1 21 41
CTGCTGAGCCATGGCTGAAGGGGAAATCACCACCTTCACAGCCCTGACCGAGAAGTTTAA
MetAlaGluGlyGluIleThrThrPheThrAlaLeuThrGluLysPheAs

61 81 101
TCTGCCTCCAGGGAATTACAAGAAGCCCAACTCCTCTACTGTAGCAACGGGGGCCACTT
nLeuProProGlyAsnTyrLysLysProLysLeuLeuTyrCysSerAsnGlyGlyHisPh

121 141 161
CCTGAGGATCCTTCCGGATGGCAGTGGATGGGACAAGGGACAGGACGACACACAT
eLeuArgIleLeuProAspGlyThrValAspGlyThrArgAspArgSerAspGlnHisIl

181 201 221
TCAGCTGCAGCTCAGTGCAGAAAGCGTGGGGAGGTGTATATAAAGAGTACCGAGACTGG
eGlnLeuGlnLeuSerAlaGluSerValGlyGluValTyrIleLysSerThrGluThrGl

241 261 281
CCAGTACTTGGCCATGGACACCGACGGGCTTTTATACGGCTCAGACACCAATGAGGA
yGlnTyrLeuAlaMetAspThrAspGlyLeuLeuTyrGlySerGlnThrProAsnGluGl

301 321 341
ATGTTTGTCTCGAAAGGCTGGAGGAGAACCATTACAACACCTATATATCCAAGAAGCA
uCysLeuPheLeuGluArgLeuGluGluAsnHisTyrAsnThrTyrIleSerLysLysHi

361 381 401
TGACAGAGAAGAAATGGTTTGTGGCCTCAAGAAGAAATGGGAGCTGCAACGCGGTCTCG
sAlaGluLysAsnTrpPheValGlyLeuLysLysAsnGlySerCysLysArgGlyProAr

421 441 461
GACTCACTATGGCCAGAAAGCAATCTTGTCTTCTCCCTGCCAGTCTCTTCTGATTAAAG
gThrHisTyrGlyGlnLysAlaIleLeuPheLeuProLeuProValSerSerAsp***

481
AGATCTGTTC

AFGF

1 21 41
CTGCTGAGCCATGGCTGAAGGGGAAATCACCACCTTCACAGCCCTGACCGAGAAGTTTAA
MetAlaGluGlyGluIleThrThrPheThrAlaLeuThrGluLysPheAs

61 81 101
TCTGCCTCCAGGGAATTACAAGAAGCCCAACTCCTCTACTGTAGCAACGGGGGCCACTT
nLeuProProGlyAsnTyrLysLysProLysLeuLeuTyrCysSerAsnGlyGlyHisPh

121 141 161
CCTGAGGATCCTTCCGGATGGCAGTGGATGGGACAAGGGACAGGACGACACACAC
eLeuArgIleLeuProAspGlyThrValAspGlyThrArgAspArgSerAspGlnHisTh

181 201 221
AGACACCAATGAGGAATGTTTGTCTCGAAAGGCTGGAGGAGAACCATTACAACACCT
rAspThrLys***

241 261 281
ATATATCCAAGAAGCATGCAGAGAAGAAATGGTTTGTGGCCTCAAGAAGAAATGGGAGCT

301 321 341
GCAACGCGGTCTCGGACTCACTATGGCCAGAAAGCAATCTTGTCTTCTCCCTGCCAG

361 381
TCTCTTCTGATTAAAGAGATCTGTTC

AFGF'

Figure 2. Structures of aFGF and aFGF'. DNA fragments obtained by RT-PCR were cloned into an Mp13M18 vector as described in Materials and Methods, and DNA sequence was obtained by the dideoxy chain termination method. The aFGF' cDNA lacks the entire second exon (boundaries marked by arrows in aFGF cDNA sequence) of the aFGF gene and codes for a protein of 60 amino acids.

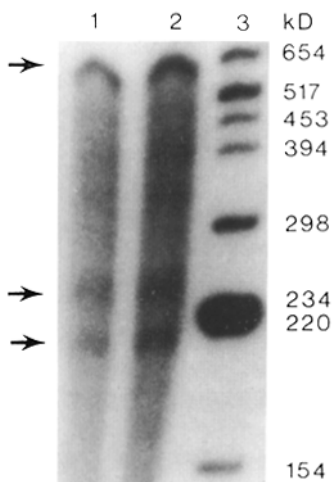


Figure 3. Ribonuclease protection assay of human lung fibroblast RNA. An aFGF riboprobe complementary to the coding region of the aFGF gene was incubated with lung fibroblast RNA and processed as described in Materials and Methods. Lanes 1 and 2 represent the results of two experiments using human lung fibroblast RNA; lane 3 contains RNA molecular mass standards. The arrows indicate the positions of the protected RNA fragments, which are 536 bp for aFGF, and 230 and 203 bp for aFGF'.

When 50 ng/ml concentrations of aFGF were assayed in the presence of increasing amounts of recombinant or synthetic aFGF', the mitogenic potential of aFGF was competitively inhibited by aFGF' (Fig. 5 B). In a control experiment, similar excess of an irrelevant protein of similar size (insulin) did not inhibit the activity of aFGF (data not shown).

The mitogenic potential of aFGF is significantly increased in the presence of heparin, to which all FGFs bind with a rather high affinity (15). Recently, it had been found that the principal site on the aFGF protein that binds to heparin is made up of an amino acid sequence encoded by exon 3 (8). As predicted by the truncated structure of aFGF', no heparin binding was detected when synthetic aFGF' was incubated with heparin sepharose (data not shown). Consequently, the minimal effect of aFGF' on fibroblast mitogenesis was not potentiated by heparin, which enhanced substantially

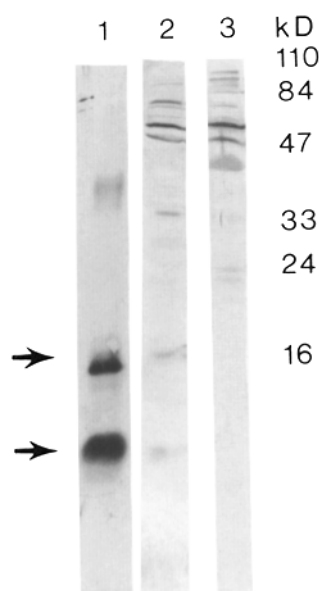


Figure 4. Immunoblot of SDS-PAGE of nuclear extracts of 10^8 human lung fibroblasts developed with anti-aFGF' antiserum. Lane 2, immune serum; lane 3 preimmune serum. Arrows indicate the positions of recombinant aFGF and aFGF' protein bands that were run in lane 1 and probed with immune serum. Positions of molecular mass standards are marked.

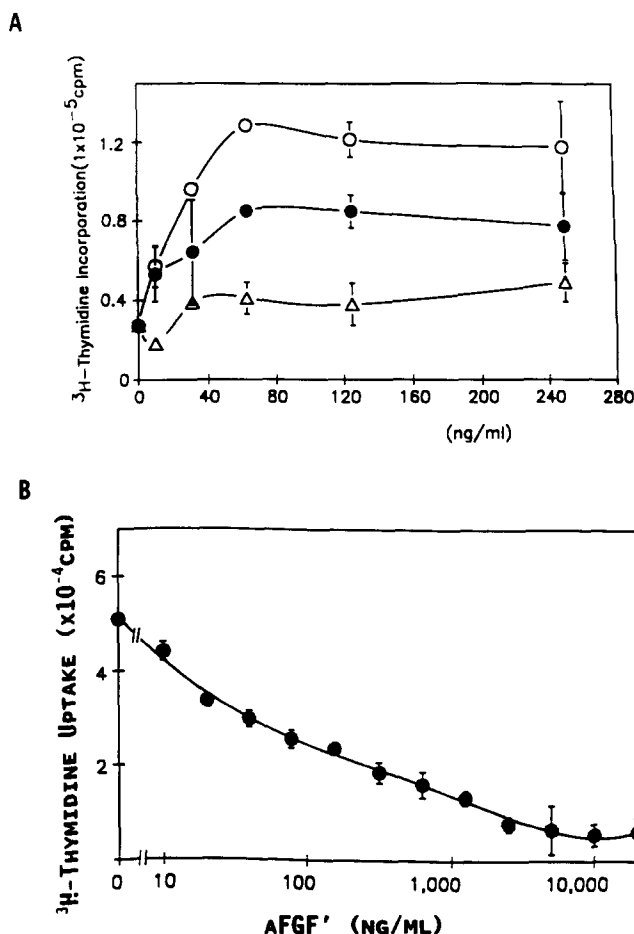


Figure 5. (A) Effects of increasing amounts of aFGF alone, aFGF + $10\times$ molar excess of aFGF', and aFGF' alone on fibroblast mitogenesis as assessed by the uptake of [3 H]thymidine into fibroblast DNA ($n = 5$). The half-maximal activity of commercial aFGF was 20 ng/ml. (O) aFGF; (●) aFGF + $10\times$ molar excess aFGF'; (Δ) aFGF'. (B) Effects of increasing amounts of aFGF' on fibroblast mitogenesis induced by 50 ng/ml aFGF.

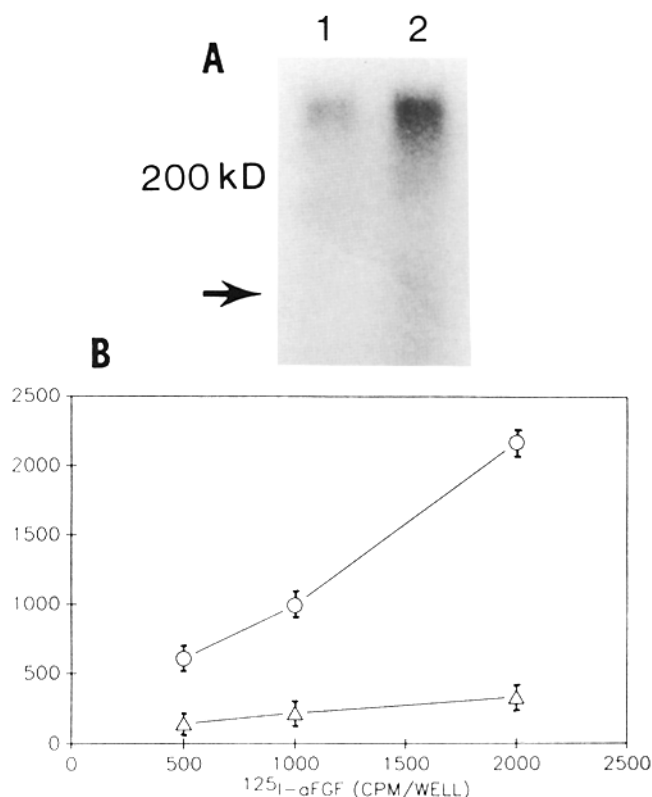


Figure 6. aFGF' binding to the FGF receptor. (A) Affinity crosslinking of 125 I-aFGF' to fibroblast receptors. Confluent BALB/c/3T3 cells were crosslinked with either 125 I-aFGF' alone (lane 2) or with a 100-fold excess of unlabeled aFGF' (lane 1). The arrow indicates the position of cross-linked receptor protein. (B) Binding assay. Confluent BALB/c/3T3 cells were incubated with radiolabeled aFGF with or without an excess amount of aFGF' ($n = 3$). (O) 125 I-aFGF; (Δ) 125 I-aFGF + 10 μ g unlabeled aFGF'.

the activity of aFGF and had no effects on the antagonistic activities of aFGF' (data not shown).

Affinity crosslinking of 125 I-aFGF' to the aFGF receptor and analysis of the radiolabeled cellular protein by SDS-PAGE and autoradiography showed a broad band with an apparent molecular mass of 145 kD (Fig. 6 A, lane 2), which was not found in the presence of an excess amount of unlabeled aFGF' (Fig. 6 A, lane 1). The size of the labeled protein is in good agreement with that reported for the aFGF receptor on BALB/c/3T3 cells (6). Studies with 125 I-labeled aFGF revealed that binding of the full-length protein to BALB/c/3T3 cells is completely blocked with excess amounts of aFGF', supporting the notion that both forms of the protein bind to a common receptor on these cells (Fig. 6 B).

As the truncated aFGF' does not induce mitogenesis of fibroblasts, we next investigated the capacity of aFGF' to evoke signals that couple aFGF receptor occupancy to cellular responses. Others have shown that aFGF increases *c-fos* expression in 3T3 cells, as assessed by Northern blot analyses (8). Only full-length aFGF was found to induce *c-fos* expression, whereas an equimolar amount of aFGF' lacked this activity at various time points after stimulation (Fig. 7). Analysis of

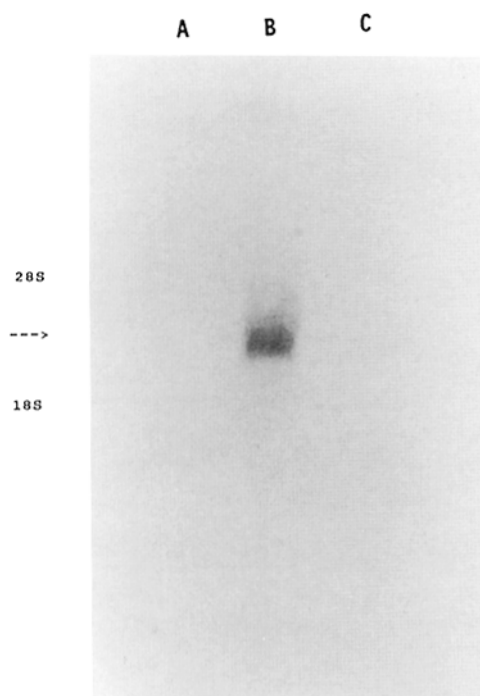


Figure 7. Differential induction of *c-fos* mRNA in fibroblasts by aFGF and aFGF'. Northern blot analysis of total RNA isolated from BALB/c/3T3 cells stimulated with either 50 ng/ml aFGF (lane B), 18.6 ng/ml aFGF' (lane C), or medium alone (lane A). Only the result of 30 min after stimulation is shown.

the pattern of tyrosine-phosphorylated proteins (7) in extracts of BALB/c/3T3 cells after stimulation with aFGF and aFGF' (data not shown) demonstrated that aFGF' failed to elicit the modification of several proteins characteristically of aFGF. The results of both types of studies indicate that aFGF' does not induce biochemical or cellular events typically evoked by aFGF. aFGF' apparently is missing structures of the full-length aFGF that are essential for receptor activation and subsequent productive transduction of signals to cells (8).

Overexpression of aFGF'. Transfectants of aFGF and bFGF in 3T3 cells have been shown to induce a transformed phenotype in these cells that is characterized by excessive growth, foci formation, and the loss of contact inhibition (16, 17). To study the function of the alternate aFGF' on growth and transformation in fibroblasts, stable transfectants were generated of aFGF' and aFGF as well as cotransfectants of aFGF and aFGF'. BALB/c/3T3 fibroblasts were transfected with either a recombinant plasmid expressing aFGF or aFGF', or with both plasmids. Fig. 8 shows the result of an RNase protection assay of several transfectants that were obtained. Whereas no bands are visible for 3T3 cells (small amounts of endogenous aFGF mRNA in 3T3 cells were detected by the RT-PCR methodology; data not shown) or cells transfected with a plasmid with no insert (RC1), all the other transfectant cells result in either a protected RNA species of 504 bp for aFGF (L4, L5), two protected RNA fragments of 230 and 171 bp for aFGF' (S5, S7, S8), or all three RNA

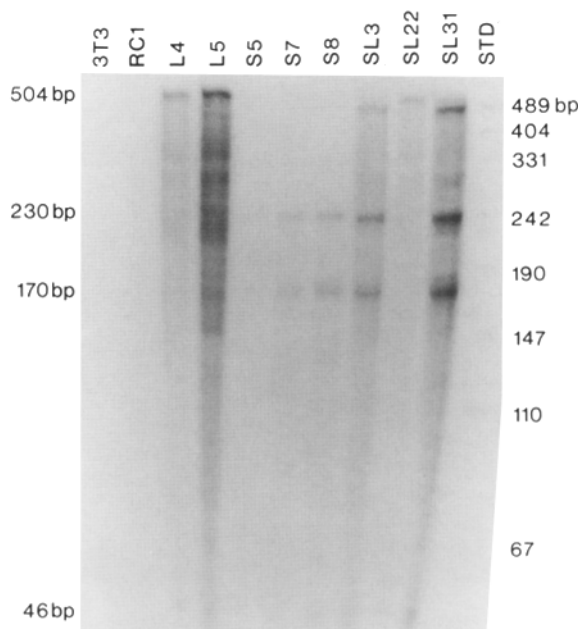


Figure 8. RNase protection assay of transfectants. The same aFGF riboprobe that was used in Fig. 3, which is complementary to the coding region of the aFGF gene, was incubated with transfectant RNA and processed as described in Materials and Methods. (3T3) BALB/c/3T3 cells; (RC1) transfectant with plasmid with no insert; (L4, L5) aFGF transfectants; (S5, S7, S8) aFGF' transfectants; (SL3, SL22, SL31) aFGF'/aFGF cotransfectants. Protected RNA species are 504 bp for aFGF and 230 and 171 bp for aFGF', respectively. The protected 46-bp γ -actin RNA fragment is shown at the bottom of the gel. RNA molecular mass standards are shown in the right lane.

Table 1. Proliferation Assay of Transfected Cell Lines

Cell line	Cell number ($n = 2$)	
	Exp. 1	Exp. 2
3T3	121,200	137,400
RC1	135,900	134,700
S5 (aFGF')	71,100	88,800
S7 (aFGF')	66,000	59,400
S8 (aFGF')	56,400	54,300
L4 (aFGF)	260,700	289,500
L5 (aFGF)	382,800	380,100
SL3 (aFGF'/aFGF)	110,400	105,300
SL22 (aFGF'/aFGF)	96,000	80,100
SL31 (aFGF'/aFGF)	68,700	77,100

30,000 cells were grown for 5 d, trypsinized, and counted. 3T3 (BALB/c/3T3 cells); RC1 (cells transfected with plasmid with no insert); S5, S7, S8 (aFGF' transfectants); L4, L5 (aFGF transfectants); SL3, SL22, SL31 (aFGF'/aFGF cotransfectants).

species in the cotransfectants (SL3, SL22, SL31). In the latter cells, a higher level of aFGF' RNA as compared with aFGF RNA is found in SL3 (aFGF'/aFGF, 2:1) and SL31 cotransfectants (aFGF'/aFGF, 3:1), whereas the reverse is true for the SL22 transfectant cell line (aFGF'/aFGF, 1:2). A 46-bp protected γ -actin RNA served as an internal control and revealed that equal amounts of total RNA had been used in the assay. Results from Table 1 show that cells expressing aFGF (L4, L5) grow to a much higher density than untransfected cells (3T3) or cells transfected with a plasmid with no insert (RC1). In the transfectants overexpressing aFGF' (S5, S7, S8) and cotransfectant cell lines (SL3, SL22, SL31), however, reduced growth rates were found as compared with untransfected cells. Furthermore, whereas aFGF transfectants were characterized by a disorganized, multilayered growth, all the other transfectants, including aFGF' transfectants and cotransfectants, revealed a normal monolayered growth pattern.

Discussion

We describe here the discovery of an alternate mRNA form of the aFGF gene that encodes a truncated aFGF protein and can be found in several lines of cultured human cells. The mRNA of aFGF' is missing the entire nucleotide sequence of the second coding exon of the aFGF gene, as assessed by the RT-PCR technique. The failure to detect aFGF' mRNA previously is very likely due to the inability to resolve a species only 100 bp smaller on routine Northern blots designed for studies of large mRNAs. Further, the very complex mRNA pattern of the aFGF gene (12) is likely to prevent detection of another mRNA. PCR with primers flanking the coding region of aFGF generates large quantities of the respective cDNA species of aFGF and aFGF', which are readily separated on agarose gels (Fig. 1). Likewise, there are two reasons why the aFGF' protein escaped attention previously. Most of the available antibodies against aFGF are directed against epitopes that are encoded by exons 2 and 3, and hence will not recognize the alternate aFGF' form, which is encoded almost exclusively by exon 1. Furthermore, the detection of proteins as small as aFGF' by SDS-PAGE and Western blot analysis required higher percentage acrylamide gels to prevent loss of such small proteins that otherwise run off and elute out of the gel or wash off the membrane during processing of Western blots (18). We have found that prolonged incubation and washing steps indeed reduced the signal of the blotted aFGF' protein considerably and also made it difficult to assess the exact ratio of aFGF and aFGF' in the cells. Consistent with earlier observations of the nuclear localization of aFGF (19), we also found that aFGF' can be detected in the nucleus of cells, and enrichment of nuclear proteins provided the only way of detection of aFGF' protein. Functional studies that were carried out with aFGF' demonstrated

that the protein acts like an antagonist for the full-length form, a potent mitogen for mesoderm- and neuroectoderm-derived cells.

The binding of aFGF' to the aFGF receptor is consistent with studies of bFGF, which assigned the binding domain to amino acid sequences encoded by exon 1 (8). Studies by others had revealed that the heparin-binding site of aFGF is caused by a sequence of basic amino acids encoded by exon 3 of the aFGF gene (8). The structures encoded by exon 3 are missing in the alternate form due to the early termination of translation. The structures of aFGF encoded by exons 2 and 3, which are deleted from aFGF', appear to be important for receptor activation, since aFGF' lacks mitogenic activity, and antagonizes the effects of aFGF. This antagonistic effect was found in mitogenesis assays with murine fibroblasts and human endothelial cells. Similar effects were also found when aFGF' was overexpressed either by itself or together with aFGF in murine fibroblasts that express only small amounts of endogenous aFGF and aFGF', as detected by the RT-PCR methodology. aFGF' transfectant cell lines showed reduced growth rates as compared with aFGF transfectant or untransfected cells. These effects are consistent with an internal autocrine mechanism that has been proposed as a way for cellular transformation (20). In contrast to the external autocrine mechanism, ligands, in this case aFGF and aFGF', and their specific receptor interact with each other inside the cell, for instance, along the secretory pathway, to generate a stimulatory signal (20). Based on this model, we assume that in the cotransfectant cell lines, aFGF' competitively inhibits the transforming activities of aFGF.

The discovery of a natural antagonist for the potent mitogen aFGF, which appears to be generated by alternate splicing of pre-mRNA, provides a novel mechanism for control of cell growth stimulated by polypeptide growth factors. Since the presumed nuclear translocation sequence of aFGF is encoded by exon 1 (19), and hence preserved in the aFGF' structure, it is conceivable that the alternate form also gets translocated into the nucleus of the cells, which is consistent with our Western blot results. There is no current evidence for regulation of cell growth by aFGF' *in vivo*. However, it is attractive to assume that initiation of a proliferative response by aFGF also results in upregulation of the expression of aFGF', subsequent blocking of binding sites on the FGF receptor by the antagonist, and downregulation of the proliferative response of the target cells. To study the modulation of the mitogenic effect of aFGF by aFGF' in cellular growth, we are currently investigating if there are differences in the ratio of the two proteins at different stages of the cell cycle. As both forms of aFGF are made by the same cell, it is conceivable that this mechanism is important in normal regulation of cell growth and differentiation, and may be impaired in cancerous growth or abnormal tissue fibrosis.

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